# Avian Two-Generation Toxicity Test Detailed Review Paper

NACEPT

Endocrine Disruptor Methods Validation Subcommittee

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# Detailed Review Paper:

# AVIAN TWO-GENERATION TOXICITY TEST

**WORK PERFORMED BY**

Battelle
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**On behalf of the United States Environmental Protection Agency**

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# OVERVIEW AND SCIENTIFIC BASIS OF AVIAN TWO-GENERATION TESTS

- Hormonal control of sexual differentiation in birds differs from that of mammals
- Birds lack fetoprotein
- Oviparity in birds allows retention of compounds
- Current protocols
  - are not sufficiently robust to differentiate endocrine disrupting chemicals from other reproductive or developmental toxins
  - are not designed to determine long-term effects of *in ovo* exposure
  - Do not assess effects at the 4 critical life stages that could be sensitive to endocrine disruption

# Test Species

- Japanese Quail (*Coturnix japonica*)
- Bobwhite (*Colinus virginianus*)
  - terrestrial habit
  - accepted model for toxicity tests
  - adaptable to laboratory conditions
  - indeterminate layer
  - precocial young

# Japanese Quail
## Family Phasianidae (Pheasants/Partridges)

- Old World quail
- Rapid incubation and maturation
  - Reproductively mature in 6 to 8 weeks
- Sexually dimorphic
- Can be kept in breeding condition all year (optimal 5 to 6 months)
- High rate of egg production (300 eggs per year)
- Peak production within 2-3 weeks of onset of lay
- Highly adaptable to battery cages
- Endocrine and behavioral patterns are well characterized
- Large number of cultivated strains

# Bobwhite

## Family Odontophoridae (New World Quails)

- Indigenous to North and South America
- Reproductively mature in 24 weeks
- sexually dimorphic
- Adapts well to laboratory
- Highly photsensitive
- females produce about 1 egg per day
- Peak egg production at 6 weeks after onset of lay
- Little deliberate selective breeding

# Advantages

## Japanese Quail

- Endocrine, behavior patterns known
- Small bird; occupies 230 $cm^2$ per bird
- Reach sexual maturity by 6 weeks
- Prolific layer
- Early maturity (36 days male, 42 days female)
- Short incubation period (16-17 days)
- Males are aggressive breeders
- Males maintain high fertility (90%)
- Adapts well to breeder cages
- Produce large egg (8% of body wt)
- Naturally hardy in laboratory
- Highly photosensitive
- Dimorphism of plumage by 3 weeks
- Male cloacal gland
- History of toxicity testin
- Spermatogenesis is well characterized

## Bobwhite

- Less domesticated, wild type
- Small bird; occupies 400-900 $cm^2$ per bird
- Prolific layer (somewhat less than Japanese quail)
- Males are aggressive breeders
- Males maintain high fertility (95%)
- Adapts well to laboratory
- Produces large egg (8% - 10.5% of body wt)
- Highly photosensitive
- Populations not prone to photperiodic drift
- Dimorphism of plumage color
- History of use in toxicity testing
- More yolk per egg (39.8%) by weight) compared with Japanese quail (31.9%)

# Disadvantages

Japanese Quail

- Inbreeding not tolerated (impaired fertility)
- Strains differ in body weight, maturation rate, egg production, lipid deposition in body and egg
- Populations can show marked photoperiodic drift with large variability in reproductive response
- Food wastage making food consumption measurement difficult
- Most strains have colored eggshells that are difficult to candle (some white egg strains available)
- Less yolk per egg compared to bobwhite
- Little used in U.S. for toxicity tests

Bobwhite

- Long incubation period (23 days)
- Long maturation period (24 months)
- Food wastage making food consumption measurement difficult
- Sex cannot be distinguished by plumage until 12 weeks of age
- Lack cloacal gland
- Spermatogenesis not characterized

# Exposure Considerations

- Exposure of parental (P1) generation
  - Pre-egg laying
    - Risk of loss of statistical power due to infertile pairs
    - Effects on sexual maturation possible
    - Bioaccumulation of test substance
  - post-initiation of laying (proven breeders)
    - Statistical advantages
      - removing nonproductive birds before exposure
      - Use pretreatment measures as covariates
    - Reduction in cost from reduced exposure period
    - Rapidly of manifestation of reproductive effects observable

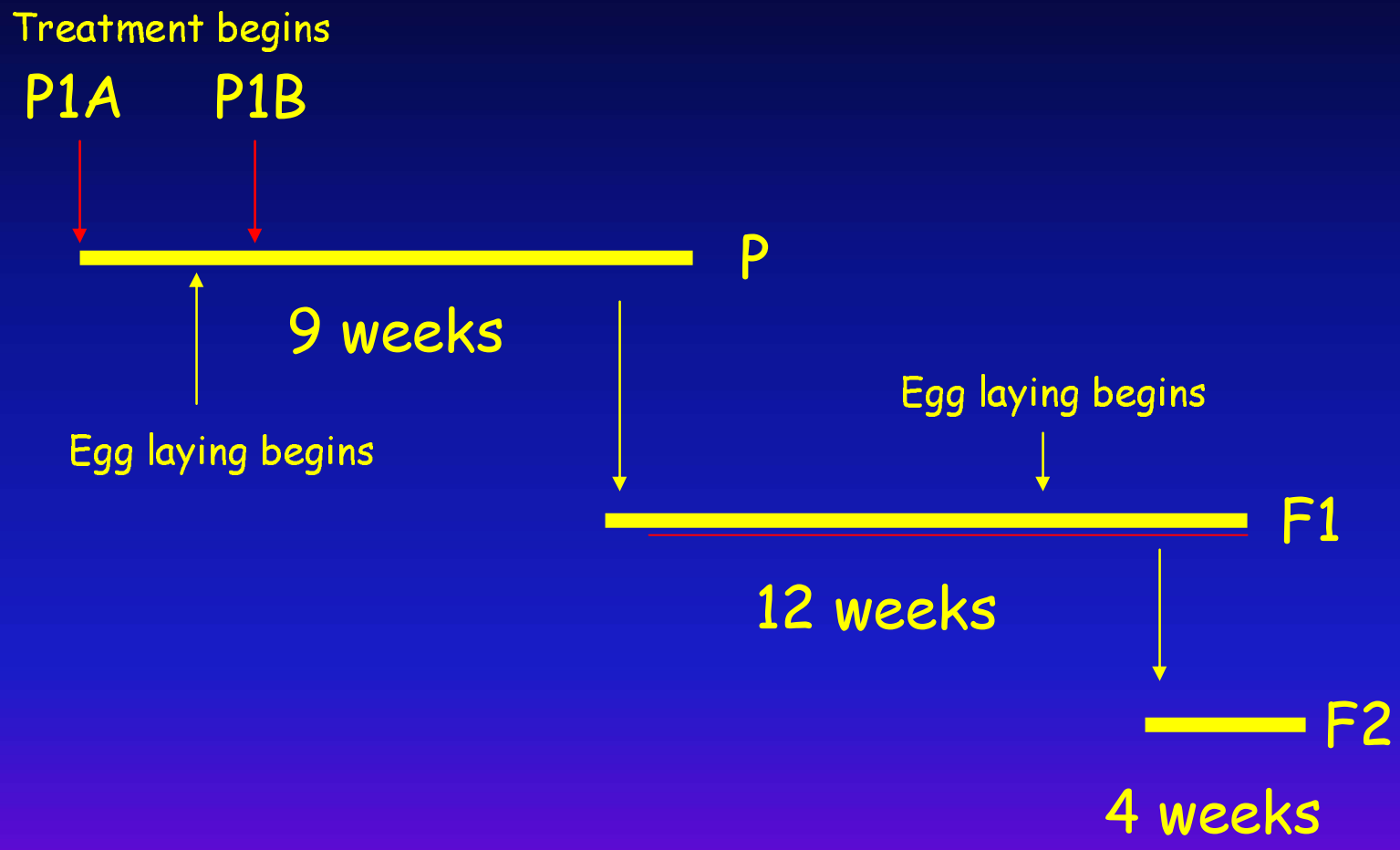
# Exposure Considerations (cont.)

- Exposure of offspring (F1) of parents
  - No exposure (*in ovo* exposure only)
    - Avoids masking of endocrine-mediated effects by high mortality of chicks from direct toxicity
  - Exposure from hatch through egg-laying
    - Worse case scenario
    - Allows observation of effects at all life stages
    - Mortality of chicks could mask endocrine-mediated effects
- F2 chicks not exposed

# Exposure Considerations (cont.)

- Combined exposure scenarios for P1 and F1
  - Maximize attributes, minimize disadvantages
    - Expose maximum number of reproductive processes
    - Expose the maximum number of life stages
    - Increase power of test
    - Not mask endocrine-related effects/not confound interpretation of results
    - Worst-case environmental exposure
    - Time and cost-effectiveness
  - Dosing Study

# Avian Dosing Study Schematic



# Routes of Administration of Chemical

- **Food**
  - More natural exposure, but greater than in wild
  - Avoids intermittent high body loading
  - Detect food avoidance/anorexia consequences
  - Low labor cost for administration
  - Dose estimation not precise
  - Costs verifying concentration and stability high
  - Effect of natural endocrine active substances in feed unknown
- **Water**
  - Ecologically relevant route
  - Dispersion of chemical more easily achieved than in diet
  - Not used for chemicals of low solubility in water
  - Evaporation may concentrate chemical
  - Water spillage more serious than feed spillage

# Route of Administration (cont.)

- **Bolus**
  - Most accurate dose estimate
  - Best for unstable or volatile chemicals
  - Low analytical costs to verify dose
  - Handling stress
  - Greater absorption rate, saturation of hepatic enzymes
  - Can result in lower tolerated test concentrations
  - Intubation trauma
  - Regurgitation of emetic compounds
  - High labor cost of dosing

# Dosing Options

- Constant dietary concentration
  - More directly comparable to environmental concentrations
  - Young can receive higher doses than parents
- Constant daily dosage
  - Can compare effects between life stages and species of different body size
- Both methods are highly artificial
  - Determine by data needs of risk assessment process

# Statistical Approaches

- Multiple comparison methods-NOAEC
  - Robust to non-normal errors
  - Sensitive to loss of replicates
  - Requires greater number of test animals
  - NOEC/LOEC limited to concentrations on test
- Regression methods-ECx, BMD
  - Less affected by loss of replicates
  - Estimate a dose-response curve
  - Estimation of ECx not limited to test concentraions
  - Assess time-series, time lag between exposure and response

# Fitness Endpoints

- Growth Rate
- Food Consumption
- Measures of Reproductive Performance
  - Fecundity
    - maximum production period
  - Fertilization Success
  - Separation of gender-specific effects
    - Fertility trials
    - Gamete Viability
      - Sperm motility and morphology
      - Sperm mobility
      - Interaction of sperm with egg perivitelline layers

# Fitness Endpoints (cont.)

- Egg quality
  - Candling-distribution of background cracking
  - Eggshell thickness
  - Eggshell Strength
    - Puncture test
    - Compression test
    - Eggshell matrix proteins as biomarkers
- Hatching Success
- F1 and F2 post-hatch suvivorship
  - Primary, integrating production endpoint
  - High CVs

# Fitness Endpoints (cont.)

- Changes in breeding behavior
  - Sensitive endpoint
  - Ecologically relevant
- Neurological/CNS impairment tests
  - Integrated response to toxin
  - Open-field tests

# Physiological Endpoints

- Organ growth and morphological changes
  - Gonadosomatic index
  - Organ:brain weight index(brain, thyroid, adrenal gland)
  - Oocyte diameter
  - Oviduct differentiation
  - Developmental landmarks
    - Gross landmarks
      - Age at first egg
      - Cloacal gland size, age at foam production
      - Age of sexually dimorphic plumage development
      - Age at first crowing
      - Sternotracheal (syringeal) muscles
      - Medualary bone
    - Histopathology of juvenile and adult tissues
- Sex Ratio

# Biochemical Measures

- Biomarkers of hepatic metabolic changes
  - Vitellogenin
    - Alkaline-labile Phosphate Assay
    - RIA, ELISA
      - No universal VTG antibody available
      - Quail VTG ELISA assay recently developed
  - Circulating very low-density lipoprotein
    - Enzymatic method in development
  - Cytochrome P450 1A induction
    - EROD activity
  - Aromatase immunocytochemical assay

# Biochemical Measures (cont.)

- Plasma, egg and fecal/urate hormone levels
  - Handling stress can alter circulating steroid levels
  - Fecal/Urate sampling
    - Long history of use in conservation biology (field samples) for steroid hormones
    - Recent application in avian studies
    - Rapid extraction methods available
    - May be useful for thyroid hormones, but not tested
  - RIA, ELISA assays
    - Commercial kits for steroid and thyroid hormones applicable to birds

# CANDIDATE PROTOCOLS

## Short-Term Life Cycle Test (Proven Breeders):

- Proposal for a New Avian Reproduction Toxicity Test in Japanese Quail or Northern Bobwhite (OECD April 2000)

## Life Cycle Tests:

- Standard Practice for Conducting Reproductive Studies with Avian Species (ASTM E1062-86)
- OECD Test Guideline 206: Avian Reproduction Test(OECD 1993)
- EPA OPPTS 850.2300: Avian Reproductive Test (USEPA 1996); suggested adaptations for two-generation test added by EDSTAC (EPA 1998)

## Two-Generation Test:

- Proposal for a Avian Two-Generation Toxicity Test in Japanese Quail (OECD 1999)

# Recommended Protocol

- Preferred species: Japanese quail
- Exposure protocol: TBD based on Avian Dosing Study
- Fitness Endpoints:
  - Egg production
  - Fertility
  - Eggshell integrity
  - Embryo viability
  - Hatchability
  - Sex ratio
  - Chick health
  - Signs of toxicity

# Recommended Protocol (cont.)

- Endocrine Endpoints

  Gross morphology & histopathology
  - Organ/gland weights/bone length(chicks)
  - Organ/gland histology
  - Spermatid counts & morphology
  - Gross anomalies

  Developmental landmarks
  - Feather dimorphism
  - Cloacal gland size
  - Sexual maturation/behavior

  Plasma and fecal/urate hormones
  - Steroid hormones
  - Thyroid hormones

# Significant Data Gaps

- Lack of clear information on source of and metabolic fate of xenobiotics *in ovo*.
- The effects of anti-estrogens in juvenile and sexually mature test species.
- The effects of anti-androgens in the developing embryo or hatchling.
- The effects of thyroid hormone agonists (or thyroid stimulation) on reproduction.
- Interactive effects of endocrine-active substances, especially natural phytosteroids in diets.

# Data Gaps (cont.)

- The effects of strain differences on test outcome and interpretation: what traits are co-selected with high body weight or high fecundity; what qualities should be selected for toxicity testing in random-bred lines.
- A statistical approach for delayed effects.
- Specific information on husbandry requirements of the Japanese quail that will result in consistent results in laboratory toxicity tests. Specifically information for dealing with fear, social stress, injurious pecking, etc.

# Implementation Considerations and Future Research

- Pre-validation studies
  - To select appropriate exposure regimen, recommend a direct performance comparison of proven-breeder and pre-breeding exposure regimens combined with nontreatment and worst-case hatch-through egg-laying F1 exposure scenarios
    - Do endocrine-mediated effects occur during maturation that are overlooked when only *in ovo* exposure is considered?
    - Do compounds that are directly toxic to chicks mask detection of endocrine-mediated effects?

# Implementation/Research (cont.)

- Recommend verifying the relative sensitivity of the Japanese quail and Bobwhite in a side-by-side comparison study
- Recommend determining the effects of strain selection in Japanese quail on test outcome to minimize nontreatment variability across laboratories
- If ANOVA methods are to be applied to the test, recommend investigation of statistical approach for delayed effects

# Implementation/Research (cont.)

- Recommend development of T4/T3 assays in fecal/urate samples for noninvasive monitoring of thyroid function
- Recommend PCR methods for genetic sex determination be optimized for Japanese quail
- Recommend determining the interactive effects of phytosteroids in feed on test outcome
- Recommend evaluating/standardizing husbandry practices
- Recommend guidelines for histology preparation and examination of tissues

- Validation of the study design through interlaboratory comparisons

# Issues/Concerns

- Animal usage
- Value added of 2 gen v. 1 gen
- Japanese quail sensitivity
- Lack of avian assay in Tier 1
- Time delay in validation (& costs)
- Linkage with existing avian testing framework